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Prolonged allograft survival by the inhibition of costimulatory CD2 signals but not by modulation of CD48 (CD2 ligand) in the rat

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Abstract The CD2 receptor is an important costimulatory molecule in T cell activation. Its ligand CD48 in rodents is supposed to be a homologue of human CD58, because of its similarities in structure and distribution. We evaluated the immunosuppressive activity of CD2/CD48-directed therapy in vitro and in vivo for the efficacy in prolonging rat heart allograft survival in a high responder transplant model. CD2-directed monoclonal antibody (mAb) therapy significantly prolonged median survival time to 45 days ($P < 0.001$). Suppression was mediated by down-modulation of CD2 below 20 % on lymph node cells without considerable cell depletion. In contrast, CD48 mAb could not prolong graft survival. Rejection occurred in the presence of complete CD48 modulation and, therefore, despite disruption of the CD2-CD48 interaction. CD48 mAb failed to inhibit lymphocyte activation via a mitogenic pair of CD2 mAbs and inhibited mixed lymphocyte reaction (MLR) only by an unspecific mechanism. In conclusion, our results suggest a negative regulatory signal transduction by inhibitory CD2 mAbs and argue against a pivotal role of mere disruption of the CD2-CD48 interaction in CD2-mediated immunosuppression.

Key words Transplantation · Graft survival · Antibody therapy · Costimulatory activation signals · Receptor-ligand binding

Introduction

Recently, it was shown in a rat heart transplant model that inhibition of costimulatory CD2 signals is highly immunosuppressive with induction of indefinite allograft survival [8]. CD2 is not only an important cell adhesion molecule that regulates cell to cell interaction and cell locomotion [5], but can also activate T lymphocytes via the "alternative pathway" [14]. The ligand of CD2 in humans is CD58 (LFA-3) that is expressed widely on haematopoietic and various non-haematopoietic cells including endothelial cells [5, 17]. So far, no equivalent of CD58 has been found in rodents. Kato et al. [9] have shown murine CD2 to bind CD48. van der Merwe et al. [12] has recently demonstrated a CD2-CD48 interaction in the rat by rosetting rat CD2 transfected COS-7 cells with soluble CD48-CD4-coated polystyrene Dynabeads. However, binding is of low affinity and can be completely blocked by a CD2 (OX34) and CD48 (OX45) mAb, respectively. CD48 is thought to be the CD58 homologue in rodents because of its similar distribution [2] and structural homology to the extracellular part of CD58 [10].

In the present study, we report on the in vivo and in vitro efficacy of a CD48 mAb (OX45) in the rat alone or in combination with an inhibitory CD2 mAb (OX34). Although a CD48 mAb has recently been used successfully in a murine transplant model [16] and CD48 is supposed to be the ligand of CD2 in the rat [13], we show that rat CD48 mAb does not produce any specific immunosuppression in vitro and in vivo. Based on the given results, we hypothesize that the in-

Table 1 Effect of CD2 and/or CD48 mAb therapy on rat cardiac allograft survival. The specified amount of mAb was i.v. injected into male Lew rats (220–250 g) that received a heterotopic cardiac transplant from DA rats

Monoclonal antibody (mAb)	Number	Total amount of mAb (mg)	Treatment (days)	Graft survival[a] (days)	Median graft survival time (days)
Control	16	–	–	6 (×4), 7 (×11), 8	7
OX34	6	2	0,1	35, 36, 36, 55, 71, 74	45.5
OX45	6	2	0,1	7, 7, 7, 7, 7, 7	7
OX45	6	6	0,1	7, 7, 7, 7, 7, 7	7
OX45	6	12	0–7	7, 7, 7, 7, 7, 7	7
OX34 + OX45	6	2	0,1	36, 42, 47, 49, 54, 65	48

[a] Complete rejection was defined as cessation of palpable heart beat and electrical activity in the ECG

hibitory CD2 mAb OX34 transduces a negative signal as compared to CD48 mAb and mere disruption of the CD2-CD48 interaction might not be the primary mechanism of CD2-mediated immunosuppression in the rat.

Materials and methods

Animals

Inbred rat strains blood group D-Agouti-RT1^a (DA) and Lewis-RT1^l (LEW) were obtained from Harlan CPB (Zeist, The Netherlands). Male rats were used, exclusively.

Heart Transplantation

We used a fully allogeneic high responder rat heart transplant model from DA (200–220 g) to LEW (220–250 g). Heterotopic intraabdominal heart transplantation was performed according to standard microsurgical techniques with an end-to-side anastomosis of the pulmonary artery to the vena cava and of the ascending aorta to the abdominal aorta. Total ischemic time was about 30 min. Rejection was defined as loss of palpable contraction and cessation of electrical activity in the ECG. For antibody therapy, a specified dose of CD2 (OX34) or CD48 (OX45) mAb was injected intravenously on two consecutive days starting immediately after transplantation.

Cells

For in vitro studies, cells were prepared and cultured from cervical and mesenteric lymph nodes (LNC) and the spleen by standard procedures [4].

Antibodies

Hybridoma cells for the following antibodies were obtained from European Collection of Animal Cell Cultures (Porton, England): OX1 (leucocyte common antigen), OX6 (MHC Class II), OX8 ($T_{c/s}$ and NK cells), OX12 (B cells), OX19 (CD5), OX21 (human C3b INA), OX34 (CD2), OX35 (T_h cells and macrophages), OX45 (CD48), OX54 (CD2), OX55 (CD2) and R73 ($\alpha\beta$TcR). Purified antibody was used for in vitro and in vivo experiments, unspecific mouse anti-human CD58 antibodies 1C7 (IgG1) and 1A10 (IgG2a) were used as controls.

Studies of lymphocyte proliferation in vitro

T cells were stimulated via the alternative pathway of CD2 by coincubation with a pair of mitogenic CD2 antibodies. 2.5×10^5 LEW-LNC and 5×10^5 syngeneic irradiated (30 Gy) spleen cells as a source of accessory cells were cultured together in the presence of a 1:1 mixture of OX54 and OX55 (20 µg/ml each final concentration) in a total volume of 200 µl/well in round-bottomed microtiter plates. 50 µl of [^{3}H]thymidine (1 µCi, Amersham, Karlsruhe, Germany) were added for the last 18 h of a 72 h incubation period before harvesting the cells on an automatic harvester (Pharmacia, Freiburg, Germany). Thymidine uptake was measured with a β-counter (Pharmacia, Freiburg, Germany).

Inhibition of allogeneic stimulation of lymphocytes by CD2 and/or CD48 antibodies was tested in a primary mixed lymphocyte reaction (MLR). 1.75×10^5 responder LEW-LNC and 2.5×10^5 irradiated (30 Gy) stimulator DA-LNC were incubated with various concentrations of CD2 and/or CD48 antibodies for 2–6 days in round-bottomed microtiter plates at 37 °C in 7.5 % CO_2. [^{3}H]Thymidine (1 µCi) was added for the last 18 h before harvesting the cells.

Flow cytometry analysis

5×10^5 LEW-LNC were incubated with 50 µl staining antibody (10 µg/ml) for 30 min at 4 °C and then washed twice with PBS. OX1 was used as a positive control, unspecific binding of OX21 as a negative control. After washing, the cells were labelled with a second-stage fluorescein-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, USA) for 15 min at 4 °C, washed twice in PBS and fixed in 1 % formaldehyde. FACS analysis was performed on an EPICS flow cytometer (Hileah, Florida, USA).

Statistical analysis

Graft survival after heart transplantation in antibody-treated animals was compared with the untreated control group in a log-rank sum test. P values < 0.05 were regarded as significant.

Results

Graft survival

The CD2 mAb OX34 and the CD48 mAb OX45 were tested in vivo for their efficacy in prolonging allograft survival (Table 1). 1 mg OX34 administered on day 0 and 1 i.v. significantly prolonged median graft survival

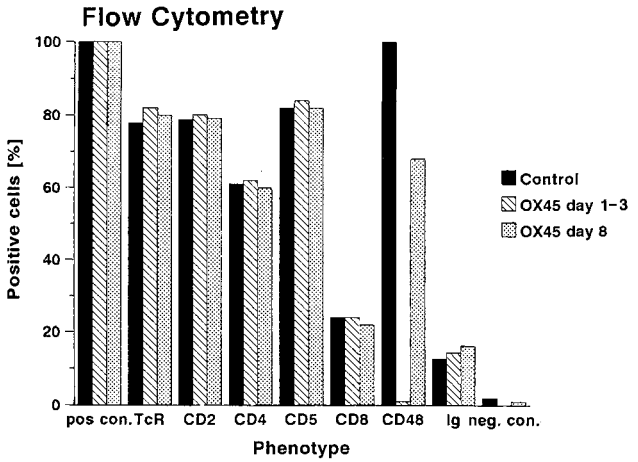

Fig. 1 Flow cytometry analysis of LNC on various days after treatment of non-transplanted LEW rats with i.v. injection 0f 3 mg OX45 on days 0 and 1. OX1 (leukocyte common antigen) was used as a positive control, OX21 (human C3b INA) as a negative control. OX45 specificly down-modulates CD48 without influencing other cell surface antigens

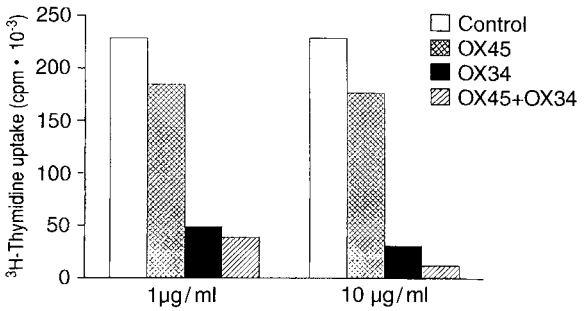

Fig. 2 CD2-mediated stimulation of 2.5×10^5 LEW-LNC by a 1:1 combination of mitogenic CD2 mAbs (OX54 + OX55; 20 µg/ml each final concentration) in the presence of 5 × 105 irradiated syngeneic spleen cells for 72 h. Results represent [^{3}H]thymidine uptake during the last 18 h period

time (MST) to 45.5 days as compared with 7 days in untreated control animals ($P < 0.001$). This immunosuppression was dose dependent, but increasing the total amount of OX34 to 5 mg (1 mg on days 0, 1, 3, 5, 7) could not produce indefinite survival (data not shown).

As CD48 is supposed to be the major ligand for CD2 in rodents, we used OX45 in the same transplant model. OX45 failed to prolong allograft survival both at an equivalent dose of 2 × 1 mg (MST = 7 days) and at a dose three times higher of 2 × 3 mg (MST = 7 days) to compensate for the wide distribution of CD48 including endothelial cells and erythrocytes. Combination of 2 × 1 mg OX45 with 2 × 1 mg OX34 did not lead to an additive or supraadditive immunosuppressive effect in vivo compared to OX34 alone (MST = 48 days; P = n.s. vs OX34).

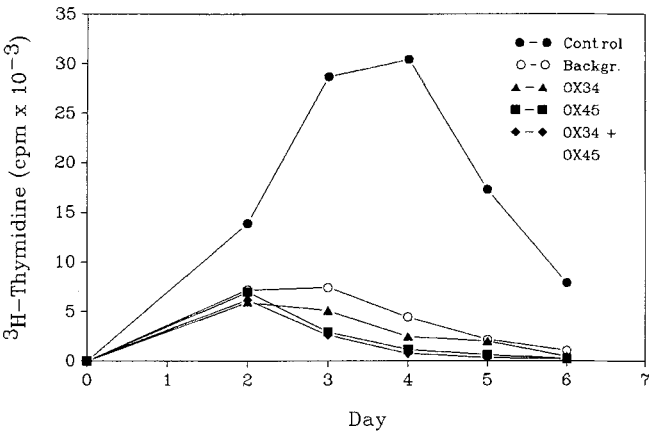

Fig. 3 Inhibition of MLR (LEW LNC vs. irradiated DA LNC) by 10 µg/ml OX34 and/or OX45 on day 2–6. Values represent [^{3}H]thymidine uptake during the last 18 h of culture.

Phenotypic analysis

Dynamic phenotypic changes of surface antigens on LNC after intravenous injection of 1 mg mAb (CD2 or CD48 mAb) on days 0 and 1 in non-transplanted rats were investigated by flow cytometry analysis at various time-points after injection until the pretreatment phenotype was reestablished. OX34 led to a rapid and almost complete (> 80 %) down-modulation of CD2 from 76 % to 13 % positive cells, residual CD2 was antibody bound. CD2 reexpression started 5 weeks after injection and was completely restored after 72 days with 68 % positive cells and no receptor-bound OX34. Circulating OX34 mAb in the serum was present until day 72. A partial and only temporary T cell depleting effect, mainly of the CD4$^+$ subset, was observed from day 14 to day 30 with a minimum of 20 % $\alpha\beta$TcR$^+$ cells (data not shown).

Application of 2 × 3 mg OX45 led to a complete down-modulation of CD48 within 1 day in the absence of any cell depletion, lasting for more than 3 days (Fig. 1). OX45 did not change expression of several other cell surface molecules, such as CD4, CD8 or TcR. Parallel to the removal of circulating mAb from serum, CD48 was partially, but functionally, reexpressed on 67 % of LNC after 8 days and fully reexpressed after 14 days. As the lack of effect of OX45 in prolonging allograft survival might be attributed to the fast biological clearance of the mAb from the circulation due to the wide distribution of CD48 along with early functional reexpression of CD48, we treated transplanted LEW rats daily from day 0–7 with a total of 12 m i.v. Even such a high-dose regimen with circulating mAb and persisting CD48 modulation for more than 8 days did not prolong allograft survival vs controls (MST = 7 days, P = n.s.).

CD2-mediated stimulation of LNC

LNC were stimulated via the CD2 receptor with a mitogenic combination of CD2 mAb (OX54 + OX55) in the presence of syngeneic irradiated spleen cells (Fig. 2). OX34 does not inhibit binding of both OX54 and OX55 [4]. While OX34 highly efficiently inhibited CD2-mediated stimulation, OX45 was not effective up to a final concentration of 100 μg/ml. Inhibition of CD2-induced proliferation by the combination of OX34 and OX45 did not differ from OX34 alone.

Allogeneic LNC stimulation in the MLR

Responder LNC were stimulated by irradiated stimulator LNC in the presence of different amounts of CD2 and/or CD48 mAb (Fig. 3). Peak proliferation was found to be on day 4 in the control MLR. The immunosuppressive effect of OX34 is fully established on days 3 and 4 with a biphasic dependence on mAb concentration. Interestingly, OX45 suppressed allogeneic LNC proliferation in this assay in a very similar manner, as seen with OX34 alone. Inhibition by OX45 is identical to the combination of OX34 and OX45.

As it is known that the inhibition of MLR by OX45 is dependent on the presence of macrophages [1], it was speculated that OX45 activates unspecific immunosuppressive activity of macrophages. We could demonstrate the non-specificity of OX45-mediated suppression of the MLR by preincubating the responder and stimulator LNC, respectively, with either OX34 or OX45 (10 μg/ml). After 24 h, cells were washed twice and stimulated in a 3 day MLR as described above. Inhibition of proliferation was exactly the same independent of whether the responder or the irradiated stimulator cells were pretreated with OX45. Inhibition was most prominent, when preincubated responder and preincubated stimulator cells were cultured together. On the contrary, the immunosuppressive effect of OX34 was exclusively restricted to pretreatment of responder cells with the CD2 mAb (data not shown).

Discussion

In the present study, we report on the in vivo efficacy of a treatment with anti-CD48 mAb OX45 after rat heart transplantation. Dynamic phenotypic changes of LNC after intravenous injection of 1 mg mAb (CD2 or CD48 mAb) on days 0 and 1 in non-transplanted rats were investigated by flow cytometry analysis on various days after injection until the pretreatment phenotype was reestablished. Even at very high doses, OX45 could not prolonged allograft survival vs untreated controls, when used alone, and had no additional immunosuppressive effect in conjunction with CD2 mAb, although it led to a dose-dependent down-modulation of CD48 without cell depletion. While the duration of allograft survival correlated with the long-lasting presence of circulating CD2 mAb in the serum and CD2 antigen modulation after antibody application, persisting down-modulation of CD48 for 7 days with the high-dose regimen of OX45 remained completely without an effect upon allograft survival. Thus, rejection occurs during ongoing OX45 therapy despite down-modulation of the ligand CD48, but does not occur in the absence of CD2 expression in the rat.

Qin et al. [16] could show prolongation of graft survival in a mouse model of non-vascularised heart transplantation. Equivalent doses of 100 μg of CD2 and CD48 mAb, respectively, were used, although CD48 has a much wider distribution comprising all haematopoietic and various non-haematopoietic cells including endothelium. While CD2 and CD48 mAb were immunosuppressive on their own, the authors demonstrated a synergistic effect with indefinite graft survival in mice treated concomitantly with CD2 and CD48 mAb. This synergistic action is in marked contrast to the findings presented in this study. These discrepant results might be explained by insufficient mAb binding affinity to rat CD48 to disrupt receptor-ligand interaction. This appears unlikely, however, as mAb binding is of much higher affinity compared with the weak CD2-CD48 interaction [13]. An alternative explanation is that there is another major functional ligand of CD2 in rats not as yet identified.

We investigated the immunosuppressive activity of OX34 and OX45, respectively, in different assays of lymphocyte activation in vitro. OX34 highly efficiently inhibited lymphocyte proliferation induced by a pair of mitogenic CD2 mAbs (OX54 + OX55), whereas OX45 failed to inhibit CD2-mediated T cell activation. The three CD2 mAbs bind to different epitopes of the CD2 molecule without cross-inhibition of binding [4]. Therefore, it might be speculated that OX34 actively transfers a negative regulatory signal via the CD2 receptor to neutralize OX54/55-mediated stimulation, provided that CD48 is the major functional ligand of CD2 in the rat. This suggestion is substantiated by the finding that mere lack of CD2 signal transduction in CD2 knock out mice does not mediate suppression [7, 11]. Transduction of negative regulatory signals by non-cross-linking CD2 mAbs has been suggested in earlier studies [3, 15, 18]. Alternatively, OX45 might be directed against an epitope of CD48, which is not involved in CD2 binding. van der Merwe et al. [13] could show, however, that COS-7 cells expressing surface CD2 specifically bind to a soluble form of CD48 (sCD48-CD4) coated on polystyrene Dynabeads and that this CD2-sCD48 interaction could be specifically blocked by OX34 as well as by OX45.

OX45 effectively suppressed lymphocyte proliferation in the allogeneic MLR, but by an unspecific mechanism as shown by preincubation experiments. This finding is in accordance with data by Arvieux et al. [1], who proposed that inhibition by OX45 is mediated via activation of non-specific suppression by accessory cells that are thought to be macrophages. The authors showed that OX45 failed to inhibit T cell activation in the presence of dendritic cells, but not when spleen cells or macrophages were used as stimulators.

In summary, CD48 is thought to be the equivalent of human CD58 in rodents because of its similar distribution and structure. Both OX34 and OX45 are known to inhibit specifically CD2-CD48 binding in vitro. As we could show, the use of CD48 mAb OX45 in vitro and in an experimental animal transplant model failed to show any specific immunosuppressive activity as opposed to the CD2 mAb OX34, indicating that negative regulatory signaling may be involved in CD2-mediated suppression rather than mere disruption of the CD2-CD48 interaction. Alternatively, an as yet unidentified ligand other than CD48 might be of major importance in binding to the CD2 receptor in the rat in vivo and in vitro.

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